Survivin mediates the anti-apoptotic effect of δ-opioid receptor stimulation in cardiomyocytes

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Summary

Survivin is known to be essential for cell division and to inhibit apoptosis during embryonic development and in adult cancerous tissues. However, the cardiovascular role of survivin is unknown. We observed that in cardiomyocytes cultured under conditions of serum and glucose deprivation (DEPV), the levels of survivin, Bcl-2 and extracellular signal-regulated kinase (ERK) were positively correlated with the anti-apoptotic action of a δ-opioid receptor agonist, [D-Ala2, D-Leu5]-enkephalin acetate (DADLE). By contrast, Bax translocation, mitochondrial membrane damage and reactive oxygen species (ROS) production were inversely correlated with the changes of survivin and Bcl-2. The use of RNA interference (RNAi) targeting survivin increased DEPV-induced cardiomyocyte apoptosis, whereas the anti-apoptotic effect of DADLE was blunted by survivin RNAi. Moreover, survivin transfection and overexpression provided protection against DEPV-induced cardiomyocyte apoptosis. Inhibition of ERK prevented the DADLE-induced decrease in apoptosis and Bax translocation, and increase in survivin and Bcl-2. DADLE-induced increase in survivin was also blunted by phosphoinositol 3-kinase (PI 3-kinase) inhibition. In conclusion, the present study provides the first direct evidence of an anti-apoptotic role of survivin mediating the anti-apoptotic effect of δ-opioid receptor activation in cardiomyocytes. ERK and PI 3-kinase were found to be upstream regulators of survivin. Mitochondrial membranes as well as ROS, Bcl-2 and Bax were also involved in this anti-apoptotic action.

Key words: Survivin, Apoptosis, δ-opioid receptor, DADLE, Cardiomyocyte

Introduction

Activation of opioid receptors has been shown to be cardioprotective by mimicking the effects of ischemic preconditioning in several experimental settings (Liang and Gross, 1999; Miki et al., 1998; Patel et al., 2002). In isolated rabbit hearts perfused on a Langendorff apparatus, morphine mimicked the effect of ischemic preconditioning in protecting the heart from being injured (Miki et al., 1998). Liang and Gross also reported a cytoprotective effect of morphine in isolated cardiomyocytes obtained from 14-day old chick embryos (Liang and Gross, 1999). Both the δ- and κ-opioid receptor subtypes have been demonstrated to mediate the cardioprotective effects of the opioids (Schultz et al., 1998; Wu et al., 1999; Zimlichman et al., 1996). These effects were assumed to be dependent on the opening of the K_{ATP} channels (Liang and Gross, 1999). In a rat model of myocardial ischemia, Patel et al. confirmed the role of the K_{ATP} channel in δ-opioid receptor activation-induced cardioprotection (Patel et al., 2002).

However, the intracellular signaling mechanisms of opioid receptor-mediated protection against cardiomyocyte apoptosis remains to be further clarified. In a rat model of myocardial infarction, extracellular signal-regulated kinase (ERK) has been shown to play a role during cardioprotection induced by stimulation of the δ_1-opioid receptor (Fryer et al., 2001a; Fryer et al., 2001b). However, Bcl-2 and Bax have been well established as intracellular regulators of the initial stage of cardiomyocyte apoptosis (Kumar et al., 2002). Bcl-2 inhibits the mitochondrial cell death pathway, whereas translocation of Bax from cytosol to mitochondria promotes cell apoptosis. The balance between Bcl-2 and Bax actions appears to determine the rate of cardiomyocyte apoptosis (Valks et al., 2003).

A small protein, survivin, interfacing cell life and death, may play a role in cardiomyocyte apoptosis. Survivin acts as a component of the chromosomal passenger complex, which is essential for cell division, and as an inhibitor of apoptosis during embryonic development and in adult cancer tissues (Altieri, 2003). A low level of survivin expression has been observed in normally differentiated tissues (Ambrosini et al., 1997). Interestingly, increased myocardial survivin expression has been reported to be associated with reduced infarct size and reduced apoptosis of the cardiomyocytes (Abbate et al., 2005; Fukuda et al., 2004; Santini et al., 2004). However, whether survivin can inhibit apoptosis of cardiomyocytes remains to be investigated. In the present study, we hypothesize that survivin inhibits cardiomyocyte apoptosis. It has to be considered that δ-opioid receptor stimulation, if used as novel therapeutic approach, may act through upregulation of survivin production and thereby provide protection against cardiomyocyte apoptosis. The apoptosis-related proteins Bcl-2 and Bax were examined during δ-opioid receptor stimulation.

Results

Ultrastructural morphology of DEPV-induced cardiomyocyte apoptosis

Under the scanning electron microscope, a normal cardiomyocyte appeared rod-shaped with a rather smooth surface (Fig. 2Aa). The serum and glucose deprivation (DEPV)-treated cardiomyocytes were characterized by detachment of the cells, which became ball-shaped (Fig. 2Ab), as well as by the formation of many small blebs (arrowhead) on cell surface (Fig. 2Ac-e) or apoptotic bodies (arrow) (Fig. 2Ae-f). The small blebs were distinct from the large bleb-shaped apoptotic bodies as described by Takemura et al. (Takemura et al., 2001). These small blebs were identified on both the cardiomyocytes with a near normal appearance (Fig. 2Ad) and the apoptotic bodies (Fig. 2Ae). The transmission electron micrographs revealed an extensive condensation of nuclear chromatin (Fig. 2Bb-e), shriveled cytoplasm (Fig. 2Bb), fragmented nuclei and apoptotic bodies (Fig. 2Bc), and phagocytosed apoptotic bodies (Fig. 2Bd), in the cardiomyocytes cultured under DEPV conditions. In certain cardiomyocytes, the remaining mitochondria and myofibrils could be identified (Fig. 2Bb). Oncotic cardiomyocytes with round nuclei, disrupted organelles and diffuse droplets were also found (Fig. 2Be).

Effects of opioid receptor stimulation on DEPV-induced cardiomyocyte apoptosis

Cardiomyocyte apoptosis was assessed by DNA ladder formation (Fig. 3A), Hoechst 33258 staining (Fig. 3B) and TUNEL staining (Fig. 3C) with fluorescence microscopy. As shown in Fig. 3A, DNA ladder formation was observed in the cardiomyocytes cultured in DEPV conditions for 12 hours. The cardiomyocytes of the control group showed a DNA smear with no detectable DNA ladder formation. In the morphine or [D-Ala2, D-Leu5]-enkephalin acetate (DADLE)-treated DEPV group, no obvious DNA ladder formation was found. The effect of morphine was attenuated by pretreatment of either naloxone or natrindole. However, the anti-apoptotic effect of morphine was not prevented by guanidinyl-naltrindole (GNTI). Likewise, the anti-apoptotic effect of DADLE was blocked by natrindole. Cardiomyocyte apoptosis was characterized by condensed chromatin and fragmented nuclei (Fig. 3B). The number of apoptotic cells were counted and analyzed on the basis of the results of TUNEL staining (Fig. 3C). Employment of DEPV for 12 hours induced a significant increase in cardiomyocyte apoptosis (36.17±5.9% versus 2.17±0.75%; $P<0.05$, $n=6$). Morphine pretreatment exerted a significant anti-apoptotic effect (19.17±1.52% vs 36.17±5.9%; $P<0.05$, $n=6$). This protective effect was attenuated by pretreatment with either the nonspecific opioid receptor antagonist naloxone (5 μmol/l) (36.67±2.58% vs 19.17±1.52%; $P<0.05$, $n=6$), or the δ-opioid receptor antagonist natrindole (5 μmol/l) (34.33±2.16% vs 19.17±1.52%; $P<0.05$, $n=6$) but not the κ-opioid receptor antagonist GNTI (5 μmol/l) (15.33±1.63% vs 19.17±1.52%; $P>0.05$, $n=6$). Moreover, the specific δ-opioid receptor agonist DADLE (10 μmol/l) exhibited an anti-apoptotic effect as well (23.5±1.64% vs 36.17±5.9%; $P<0.05$, $n=6$) This effect was blocked by natrindole (5 μmol/l) (39.17±1.72% vs 23.5±1.64%; $P<0.05$, $n=6$) (Fig. 3C. $n=6$ in each group). These data suggest that the anti-apoptotic effect of morphine was mainly mediated by activating the δ-opioid receptor. Therefore, we focused on the δ-opioid receptor-mediated pathways in the following studies.

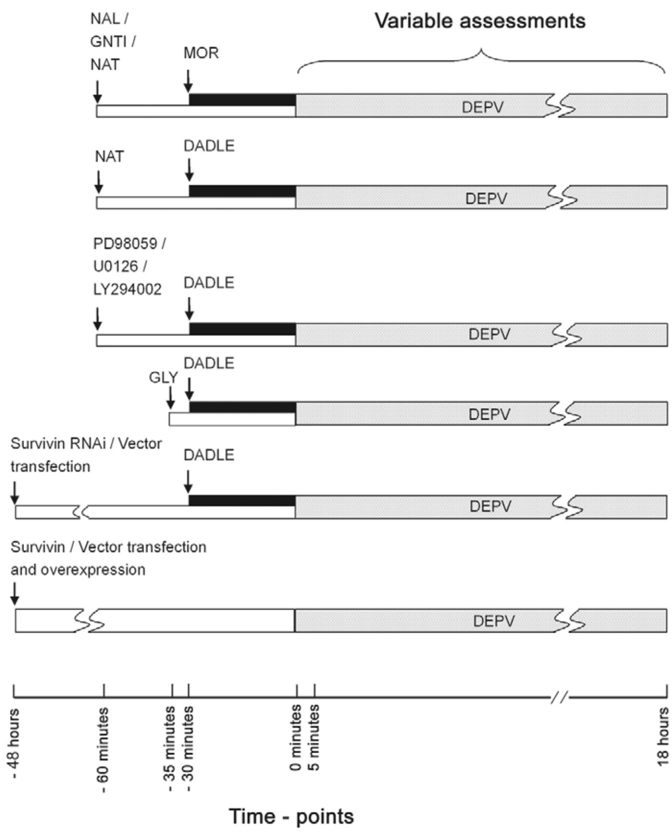

Fig. 1. Schematic illustration of the treatment protocol. DEPV, serum and glucose deprivation; MOR, morphine; NAL, naloxone; GNTI, guanidinyl-naltrindole; NAT, natrindole; DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; GLY, glybenclamide.

Effects of DADLE on DEPV-induced reactive oxygen species production and mitochondrial membrane depolarization

Reactive oxygen species (ROS) production increased in cardiomyocytes cultured under DEPV conditions in a time-dependent manner reaching a peak at 6 hours. DADLE (10 μmol/l) pretreatment inhibited this increase in ROS production, as monitored after 4, 6 and 8 hours under DEPV conditions. The most significant DADLE-induced reduction (~51%) in ROS production was observed at 6 hours (Fig. 4A).

Cardiomyocyte mitochondrial damage was assessed by examining mitochondrial membrane depolarization, which was determined by the ratio of red and green signals in the micrographs. DEPV-induced depolarization of the mitochondria appeared as a decrease in the red signals with a concomitant increase in the green signals (Fig. 4B, upper panel). Inhibition of the destruction of mitochondrial membrane potential by DADLE (10 μmol/l) was detected after 45, 60, 90 and 120 minutes under DEPV conditions.

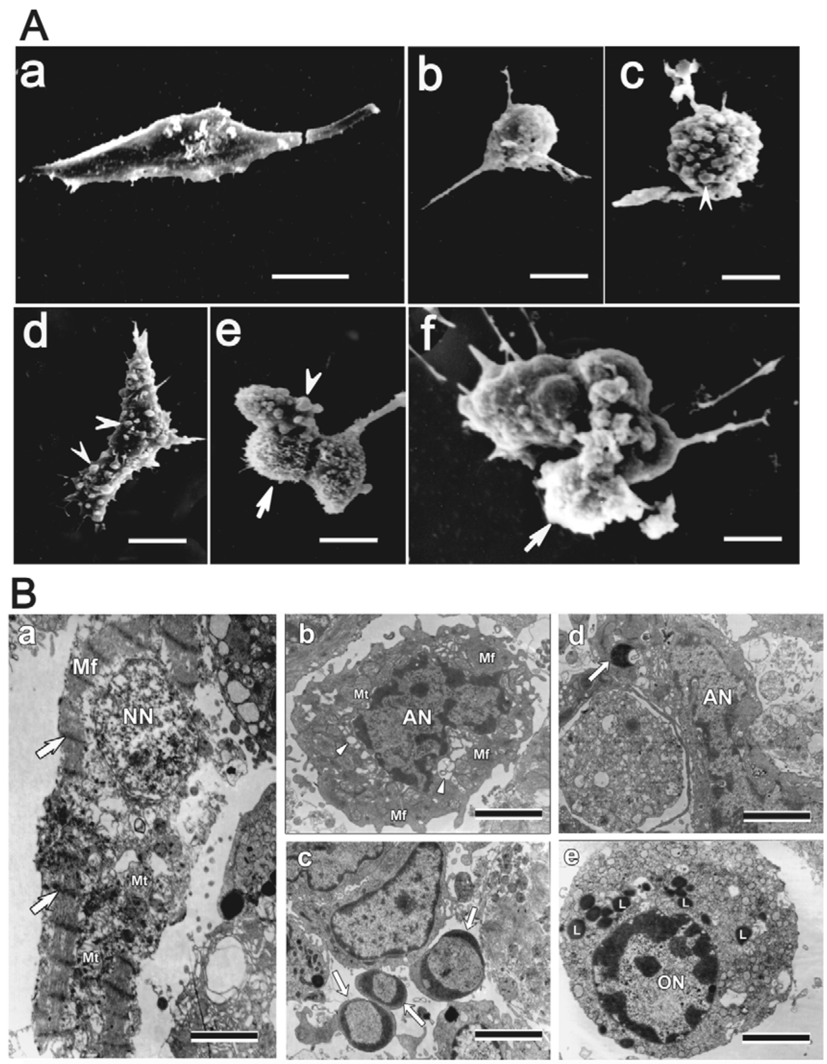

Fig. 2. Ultrastructure of DEPV-induced cardiomyocytes apoptosis. (A) Scanning electron micrographs of control cardiomyocytes (a) and those cultured under DEPV conditions (b-f). The DEPV-treated cardiomyocytes were characterized by detachment of the cell, which became spherical (b) as well as formation of many small blebs (arrowhead) on the cell surface (c-e) or apoptotic bodies (e,f; arrow). Bars, 10 μm. (B) Transmission electron micrographs of control cardiomyocytes (a) and those cultured under DEPV conditions (b-e). (a) The normal nucleus (NN), mitochondria (Mt), myofibrils (Mf) and cross striations (arrows) in a control cardiomyocyte. (b) The apoptotic nucleus (AN) with bounding condensed chromatin, shriveled cytoplasm, mitochondria (Mt), myofibrils (Mf) and a lot of vacuoles (arrowheads) in an apoptotic cardiomyocyte. (c) Apoptotic bodies (arrows) with condensed chromatin. (d) A phagocytosed apoptotic body (arrow). (e) Condensed chromatin in the round, oncotic nucleus (ON), disrupted organelles and droplets (L) in an oncotic cardiomyocyte. Bars, 2 μm.

These effects were blunted by glybenclamide (1 μmol/l; Fig. 4B, lower panel).

Time-dependent action of DADLE on ERK phosphorylation, expression of survivin and Bcl-2, as well as Bax translocation from the cytosolic to the mitochondrial fraction in cardiomyocytes cultured under DEPV conditions

DADLE caused an increase in ERK phosphorylation, detectable between 2 and 18 hours under DEPV conditions (Fig. 5A,B). Expression of survivin and Bcl-2 increased in response to DADLE treatment and was apparent from 6 to 18 hours (Fig. 5A,C,D). Translocation of Bax from the cytosol into the mitochondria was estimated by comparing the Bax levels in the cytosolic and the mitochondrial fractions. An increase in mitochondrial Bax levels accompanied by a concomitant decrease in cytosolic Bax levels suggests increased translocation. Bax protein was mainly detected in the cytosolic fraction rather than the mitochondrial fraction in the control group. DADLE-inhibited Bax translocation from the cytosol to the mitochondria was detected between 2 and 18 hours under DEPV conditions (Fig. 5A,E).

The role of DADLE-induced ERK1/2 phosphorylation in Bax translocation, Bcl-2 and survivin expression, as well as cardiomyocyte apoptosis

As shown in Fig. 6A, DADLE (10 μmol/l) caused a significant increase in ERK1/2 phosphorylation in the cardiomyocytes cultured in the DEPV condition for 6 hours (6.8±0.45 vs 1.1±0.14; $P<0.05$, $n=6$). This increase was prevented by pretreatment with either natrindole (5 μmol/l) (1.3±0.09 vs 6.8±0.45; $P<0.05$, $n=6$) or the ERK1/2 inhibitors PD98059 (50 μmol/l) (1.6±0.13 vs 6.8±0.45; $P<0.05$, $n=6$) and U0126 (10 μmol/l) (0.1±0.008 vs 6.8±0.45; $P<0.05$, $n=6$). DADLE prevented DEPV-induced increase in Bax translocation, detected at 6 hours. This effect of DADLE was abolished by either natrindole or the ERK1/2 inhibitors PD98059 and U0126 (Fig. 6B, $n=6$ in each group). Moreover, DADLE-induced increase in Bcl-2 protein was prevented by either natrindole or the ERK1/2 inhibitors PD98059 or U0126 at 6 hours (Fig. 6C). The survivin level was significantly decreased in the cardiomyocytes cultured under DEPV conditions as assessed by western blot analysis (0.37±0.06-fold, $P<0.05$ vs control, $n=4$). DADLE treatment caused a significant increase in the survivin level in the cardiomyocytes cultured under DEPV condition for 6 hours (1.29±0.12 vs 0.37±0.06; $P<0.05$,

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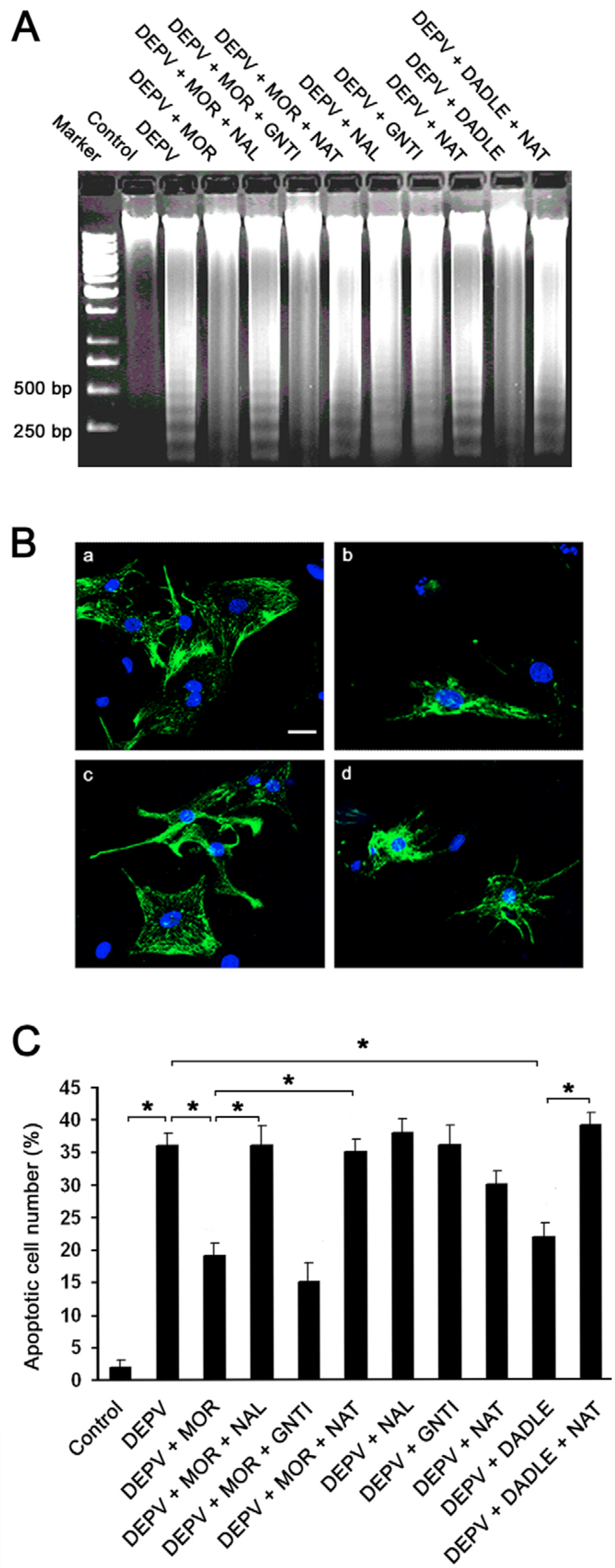

Fig. 3. Effects of opioid receptor stimulation on DEPV-induced cardiomyocyte apoptosis. (A) Representative electrophoretic analysis of nucleosomal DNA fragmentation from six experiments, showing the effect of morphine or DADLE on DEPV-induced cardiomyocyte apoptosis in the presence or absence of the nonselective-, κ-, or δ-opioid receptor antagonist. (B) Representative Hoechst 33258 staining from six experiments, showing morphine-induced protection against DEPV-induced cardiomyocyte apoptosis. a, control; b, DEPV; c, DEPV + MOR; d, DEPV + MOR + NAL. Bar, 20 μm. (C) The effect of morphine or DADLE on DEPV-induced cardiomyocyte apoptosis in presence or absence of the nonselective-, κ-, or δ-opioid receptor antagonist. Bar chart shows the percentage of apoptotic cells, as assessed by TUNEL staining, in each group (*n*=6 in each group). Values are the mean ± s.d. **P*<0.05 among the indicated groups. DEPV, serum and glucose deprivation; DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt; NAT, natrindole; MOR, morphine; NAL, naloxone; GNTI, guanidinyl-naltrindole.

n=4). This effect of DADLE was prevented by natrindole (0.41±0.09 vs 1.29±0.12; *P*<0.05, *n*=4), PD98059 (0.47±0.09 vs 1.29±0.12; *P*<0.05, *n*=4), or the PI 3-kinase inhibitor LY294002 (0.40±0.11 vs 1.29±0.12; *P*<0.05, *n*=4) (Fig. 3D). On the basis of these results, it was suggested that the survivin expression was regulated by ERK1/2-dependent and PI 3-kinase-dependent pathways following δ-opioid receptor stimulation. Moreover, the effect of DADLE in preventing DEPV-induced cardiomyocyte apoptosis as assessed by TUNEL staining was also abolished by PD98059 and U0126 at 6 hours (Fig. 6E). It was concluded that ERK1/2 mediates a DADLE-induced increase in Bcl-2 expression and a blockade of Bax translocation, as well as the anti-apoptotic effects of DADLE.

RNAi of survivin blunted DADLE-induced protection of cardiomyocytes against apoptosis

The effectiveness of survivin siRNA in suppressing endogenous survivin expression was assessed by immunofluorescent staining (Fig. 7A), real-time PCR (Fig. 7B) and western blot analysis (Fig. 7C). Successful transfection was confirmed by the green fluorescence of GFP labels conjugated with the siRNAs (Fig. 7A). Transfection of survivin siRNA and GAPDH siRNA as controls specifically knocked down the expression of their respective target genes without affecting each other's targets (Fig. 7C). Therefore, the effect of survivin RNAi was assessed by comparing its expression with the expressions of either the non-transfected or the GAPDH RNAi group.

In cardiomyocytes cultured under DEPV condition for 6 hours, survivin RNAi increased apoptosis in the absence or presence of DADLE (DEPV vs control, 39.7±2.4% vs 1.29±0.12%; DEPV + DADLE vs control, 28.7±3.3% vs 1.29±0.12%; *P*<0.05, *n*=6) (Fig. 8A,B). However, survivin RNAi did not change the rate of apoptosis in cardiomyocytes cultured under control conditions in the absence of DEPV (Fig. 8A,B). Survivin RNAi blunted the effect of DADLE in reducing apoptosis of cardiomyocytes cultured under DEPV conditions (apoptosis reduction in survivin RNAi group vs reduction in GAPDH RNAi group, 10±1.8% vs 16.7±1.3%; *P*<0.05, *n*=6) (Fig. 8C).

Survivin overexpression action on DEPV-induced apoptosis, ROS production and mitochondrial membrane depolarization

Forty-eight hours after transfection, the GFP-survivin-fused

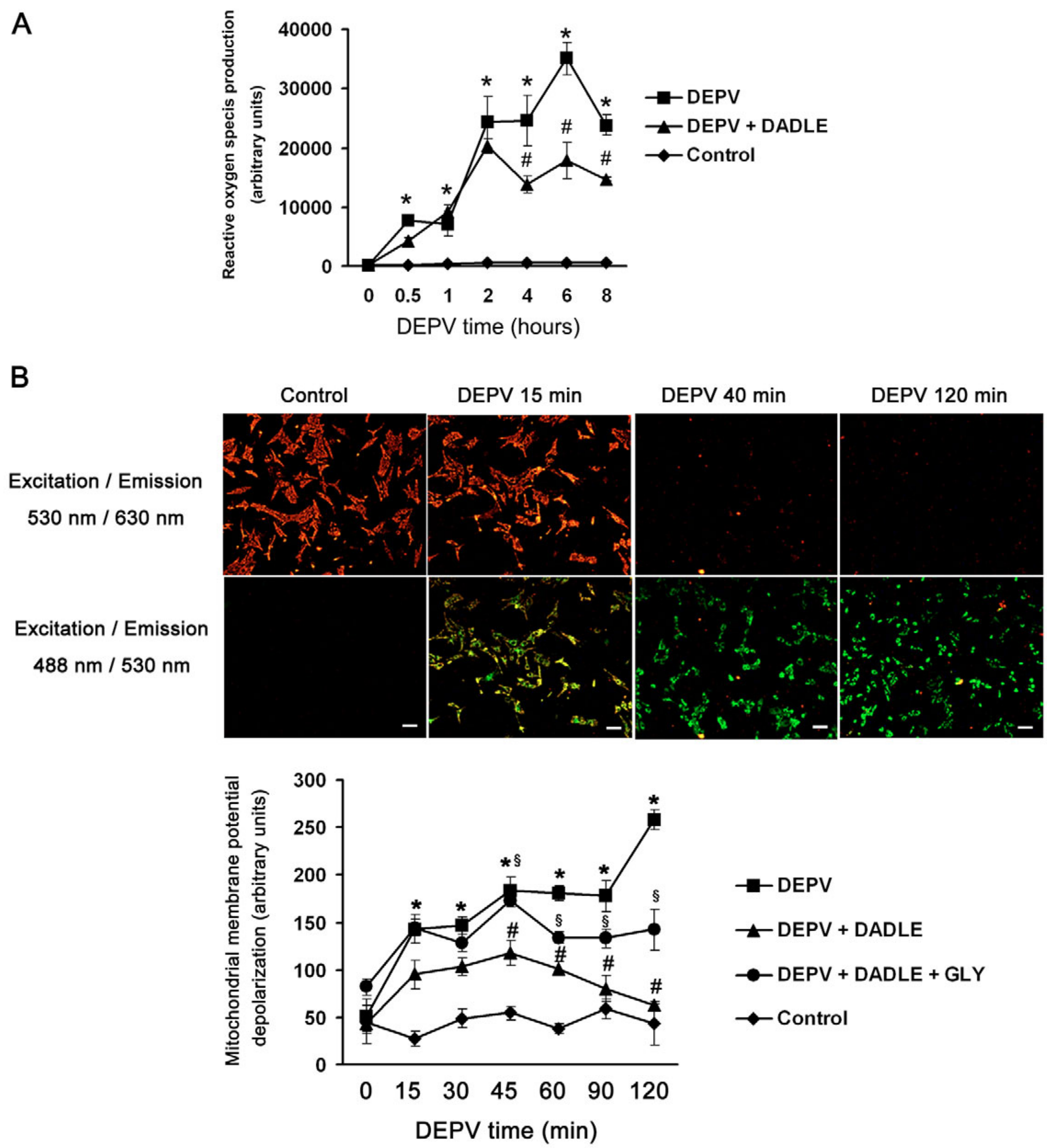

Fig. 4. (A) ROS production increased in cardiomyocytes cultured under DEPV conditions in a time-dependent manner reaching a peak at 6 hours. DADLE (10 μmol/l) pretreatment inhibited this increase in ROS production at 4, 6 and 8 hours. Values are the mean ± s.d. **P*<0.05 vs control, $^{\#}$*P*<0.05 vs DEPV. (B) Cardiomyocyte mitochondrial damage was assessed by examining mitochondrial membrane potential, which was determined by the ratio of red and green signals in the micrographs. DEPV-induced depolarization of the mitochondria appeared as a decrease in the red signals with a concomitant increase in the green signals (upper-panel). DADLE (10 μmol/l) inhibited the destruction of mitochondrial membrane potential at 45, 60, 90 and 120 minutes. These effects were blunted by 1 μmol/l glybenclamide (GLY; lower-panel). Values are the mean ± s.d. **P*<0.05 vs control; $^{\#}$*P*<0.05 vs DEPV; §*P*<0.05 vs DEPV + DADLE. DEPV, serum and glucose deprivation; DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt.

protein was overexpressed in the cardiomyocytes (Fig. 9A). Transfection and overexpression of survivin significantly inhibited apoptosis of cardiomyocytes cultured under DEPV condition for 12 hours as compared with vector-transfected control cells (19.57±2.8% vs 53.19±3.3%; *P*<0.05) (Fig. 9B,C). However, survivin overexpression had no effect on ROS production (Fig. 9D) and mitochondrial membrane potential depolarization (Fig. 9E). However, glybenclamide (1 μmol/l) was not effective in preventing the DADLE-induced increase in survivin expression (Fig. 9F).

Discussion

Morphine has been reported to protect against ischemia-reperfusion injury and reduce myocardial infarct size in rats (Patel et al., 2002), as well as rabbits (Miki et al., 1998), and protect the cardiomyocytes in the chicken embryo (Liang and Gross, 1999). It has been further demonstrated that the δ-receptor agonist DADLE and the κ-receptor agonist U-50488H could reduce myocardial infarct size and the incidence of arrhythmia in a cardiac ischemia-reperfusion model of the rat (Valtchanova-Matchouganska and Ojewole, 2003). However,

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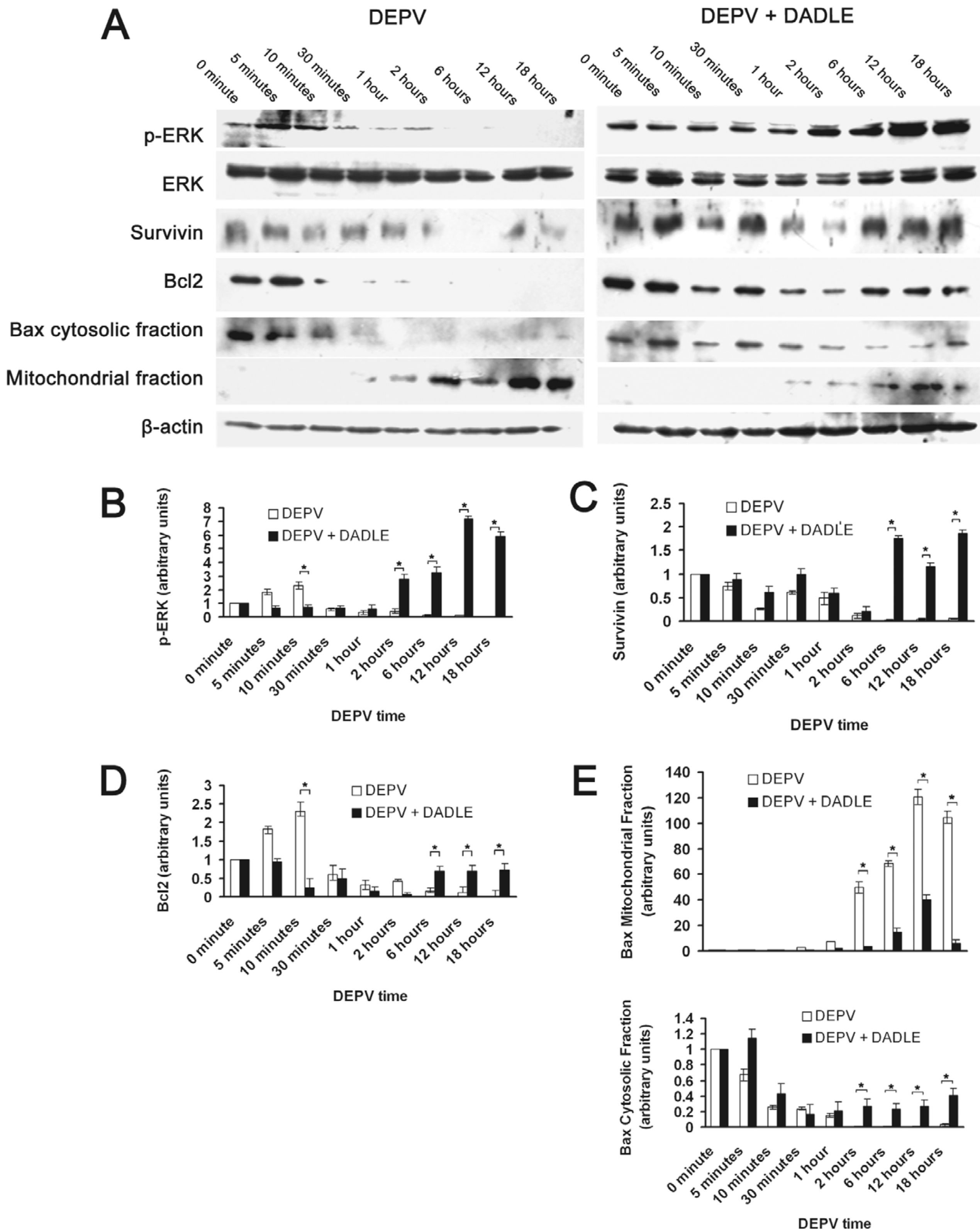

Fig. 5. Time-dependent action of DADLE on ERK phosphorylation, expression of survivin and Bcl-2, as well as Bax translocation from the cytosolic to the mitochondrial fraction in cardiomyocytes cultured under DEPV conditions. (A) Representative blots showing time course of p-ERK, ERK, survivin, Bcl-2 and the cytosolic and mitochondrial fractions of Bax in cardiomyocytes cultured under DEPV conditions with or without pretreatment with DADLE (A). (B-F) Quantification by densitometry of the bands of pERK (B), survivin (C), Bcl-2 (D), the mitochondrial fractions and the cytosolic fractions of Bax (E) (n=3 in each group). Values are the mean ± s.d. *P<0.05 between the indicated groups. DEPV, serum and glucose deprivation; DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt.

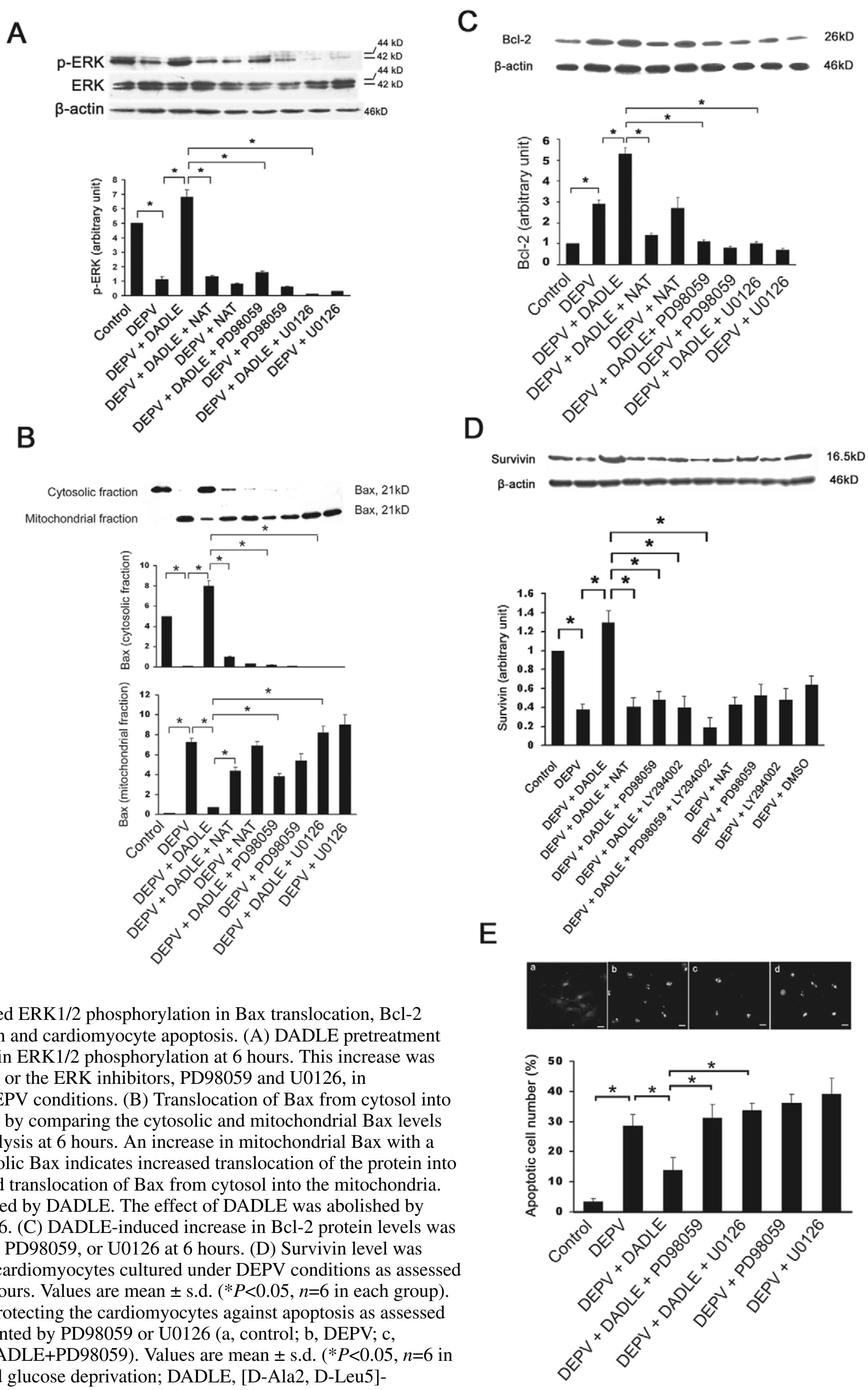

Fig. 6. Role of DADLE-induced ERK1/2 phosphorylation in Bax translocation, Bcl-2 expression, survivin expression and cardiomyocyte apoptosis. (A) DADLE pretreatment induced a significant increase in ERK1/2 phosphorylation at 6 hours. This increase was prevented by either natrindole, or the ERK inhibitors, PD98059 and U0126, in cardiomyocytes cultured in DEPV conditions. (B) Translocation of Bax from cytosol into the mitochondria was assessed by comparing the cytosolic and mitochondrial Bax levels measured by western blot analysis at 6 hours. An increase in mitochondrial Bax with a concomitant decrease in cytosolic Bax indicates increased translocation of the protein into mitochondria. DEPV increased translocation of Bax from cytosol into the mitochondria. This translocation was prevented by DADLE. The effect of DADLE was abolished by natrindole, PD98059, or U0126. (C) DADLE-induced increase in Bcl-2 protein levels was prevented by either natrindole, PD98059, or U0126 at 6 hours. (D) Survivin level was significantly decreased in the cardiomyocytes cultured under DEPV conditions as assessed by western blot analysis at 6 hours. Values are mean ± s.d. (*$P<0.05$, $n=6$ in each group). (E) The effect of DADLE in protecting the cardiomyocytes against apoptosis as assessed by TUNEL staining was prevented by PD98059 or U0126 (a, control; b, DEPV; c, DEPV+DADLE; d, DEPV+DADLE+PD98059). Values are mean ± s.d. (*$P<0.05$, $n=6$ in each group). DEPV, serum and glucose deprivation; DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt; NAT, natrindole; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio)-butadiene.

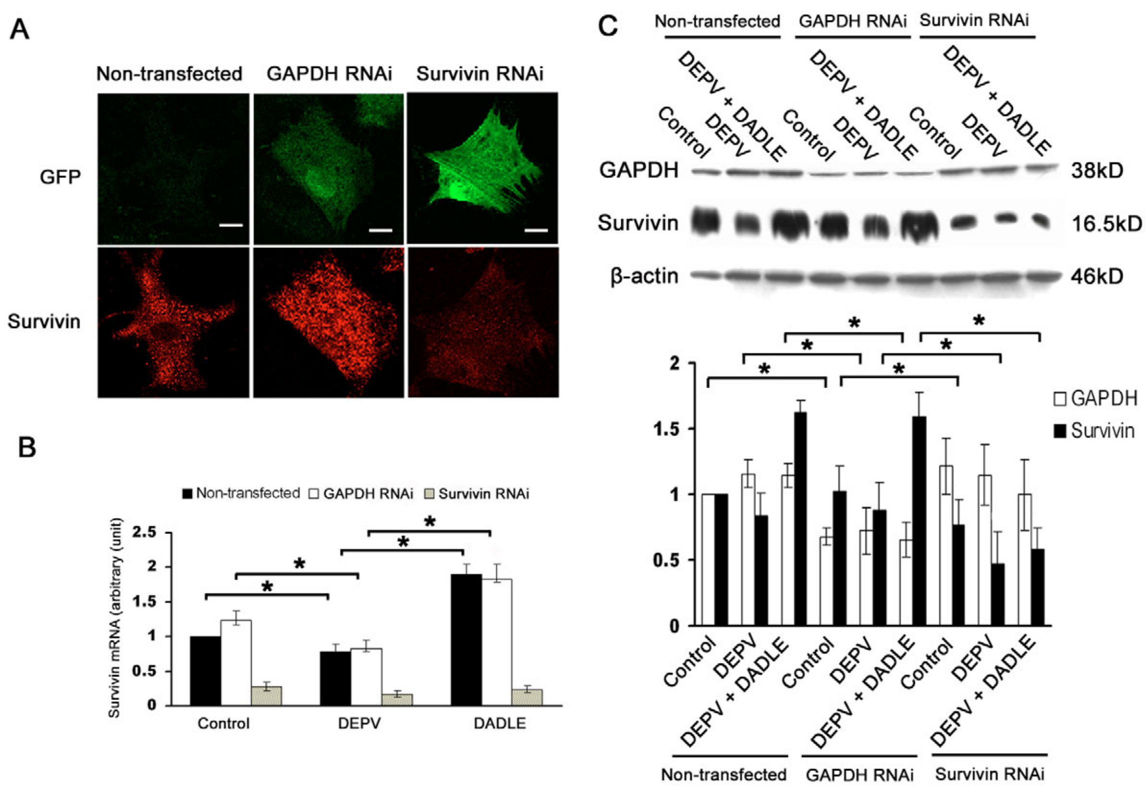

Fig. 7. Survivin RNAi abrogates DADLE-induced increase in survivin expression in cardiomyocytes. (A) Survivin expression, identified by immunofluorescent staining, was significantly decreased by transfection of survivin siRNA in cultured cardiomyocytes (red signals for survivin). Successful transfection was confirmed by the green signals of GFP labeling conjugated with the siRNAs. Micrographs are representative of four independent experiments. (B) 48 hours after transfection, mRNA transcripts of survivin as measured by real-time RT-PCR were significantly reduced by survivin RNAi in cardiomyocytes cultured under control condition, as well as in cardiomyocytes cultured under DEPV conditions with or without DADLE treatment. (C) Representative blots and quantification of survivin levels by densitometry. DADLE-induced increase in survivin levels was abrogated by transfection of survivin RNAi 72 hours after transfection. Transfection of survivin siRNA and GAPDH siRNA specifically knocked down the expression of their respective target genes without affecting each other's targets. Values are the mean ± s.d., n=6. *P<0.05. DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt; DEPV, serum and glucose deprivation; RNAi, RNA interference; siRNA, small interfering RNA.

the intracellular signaling mechanisms of the cardiac actions of opioids remain to be determined.

In the present study, we observed typical ultrastructural features of an apoptotic cell as well as the features of a cardiomyocyte. Compared with Fas antibody and actinomycin D-induced cardiomyocyte apoptosis described by Takemura et al. (Takemura et al., 2001), the apoptotic cardiomyocytes in the present study are characterized by a lot of small blebs on the surface of the cells, less myofibrils and more mitochondria. However, the oncotic cardiomyocytes in the present study contained few lipid droplets than that described by Takemura et al. (Takemura et al., 2001).

We found here that the cardioprotective effect of opioids against DEPV-induced cardiomyocyte apoptosis was mediated by the δ- but not the κ-opioid receptor. This finding is in agreement with those of Okubo et al. who reported a δ-opioid receptor-mediated effect against myocardial apoptosis in a rabbit model of ischemia-reperfusion injury (Okubo et al., 2004).

The novel finding of the present study is the observation that survivin mediated the anti-apoptotic effect of δ-opioid receptor activation in cardiomyocytes. Survivin expression has been reported to be significantly upregulated to aberrantly promote cell survival in neoplasia of various cancers (Altieri, 2003; Ambrosini et al., 1997). Whether survivin plays a role in cell survival in cardiovascular physiology and diseases is unknown. It has been reported by several groups that survivin expression was identified in the myocardium and associated with myocardial injury. In a rat model of coronary artery occlusion, ischemic preconditioning-induced reduction in infarct size was associated with increased expression of the survivin gene in the heart (Fukuda et al., 2004). In spontaneously hypertensive rats, myocardial survivin expression is inversely correlated with apoptosis (Abbate et al., 2005). In patients with acute myocardial infarction, survivin expression in the non-infarcted myocardium is inversely associated with the occurrence of dilated cardiopathy and apoptosis (Santini et al., 2004). In the present study, we demonstrated that survivin RNAi increased cardiomyocyte apoptosis whereas survivin overexpression prevented DEPV-induced cardiomyocyte apoptosis. Moreover, survivin expression was upregulated by the δ-opioid receptor agonist DADLE. The anti-apoptotic effect of DADLE was blunted by survivin RNAi. These data provide the first evidence for the anti-apoptotic role of survivin in

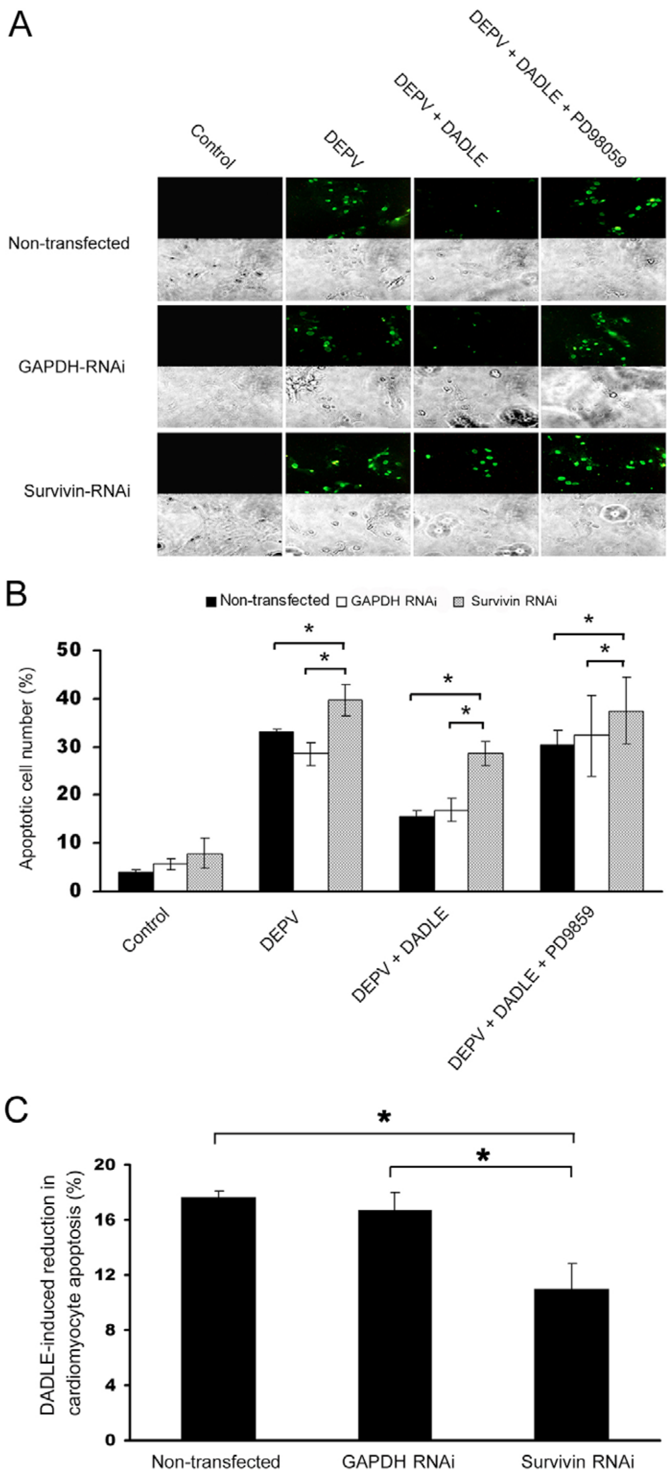

Fig. 8. Survivin RNAi blunts DADLE-induced protection of cardiomyocytes against apoptosis. (A) Representative micrographs and (B) percentage of apoptotic cells as assessed by TUNEL staining. In cardiomyocytes cultured under DEPV conditions, survivin RNAi increased apoptosis in the absence or presence of DADLE. However, survivin RNAi did not change the number of apoptotic cardiomyocytes cultured under control condition. (C) Survivin siRNA blunted the effect of DADLE in reducing apoptosis of cardiomyocytes cultured under DEPV conditions. There was a survivin RNAi-induced increase in the number of apoptotic cardiomyocytes cultured under DEPV conditions as compared with that of the non-transfected or the GAPDH RNAi group in either the absence or the presence of DADLE (A,B). However, survivin RNAi did not change the number of apoptotic cardiomyocytes cultured under control condition. DADLE-induced reduction in the cardiomyocytes transfected with survivin siRNA was significantly less than that in the non-transfected cardiomyocytes or those transfected with GAPDH siRNA (C). Values are the mean ± s.d. *$P<0.05$. $n=6$ in each group. DEPV, serum and glucose deprivation; DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt; RNAi, RNA interference; siRNA, small interfering RNA; TUNEL, TdT-mediated dUTP nick end labeling.

cardiomyocytes and enhance our understanding of the intracellular signaling mechanisms of δ-opioid receptor activation in cardiomyocytes. Indeed, the clinical significance of an anti-apoptotic role of survivin remains to be confirmed by in vivo experiments, e.g. investigation of the effect of cardio-specific transfection and overexpression of survivin on ischemia-reperfusion-induced cardiomyocyte apoptosis.

Several upstream signaling pathways have been reported to regulate survivin expression in various cancers. For example, a PI 3-kinase-specific inhibitor, but not a MEK1-specific inhibitor, was effective in decreasing survivin levels in breast cancer cells (Asanuma et al., 2005). Inhibition of ErbB2 signaling led to marked inhibition of survivin protein with subsequent apoptosis of breast cancer cells (Xia et al., 2006). Moreover, it has been suggested that inhibition of the survivin pathway is a possible selective cancer treatment strategy to induce apoptosis of cancer cells (Altieri, 2003). The results of the present study suggest that upregulation of survivin may provide a potential novel therapy in preventing cardiomyocyte apoptosis. Therefore, the upstream regulatory mechanisms of survivin in cardiomyocytes become interesting. The present study showed that the DADLE-induced increase in survivin expression was prevented by either PD98059 or LY294002. On this basis, it is probable that both ERK1/2 and PI 3-kinase are involved in regulating survivin expression. However, the precise upstream regulatory mechanisms of survivin in cardiomyocytes remain to be further elucidated.

In the present study, suppression of survivin expression by RNAi blunted, but not completely abolished the DADLE-induced anti-apoptotic effect. On this basis, we hypothesize the involvement of additional pathways. The Bcl-2 protein protects the cardiomyocytes from apoptosis by several mechanisms, such as a direct antioxidant effect, inhibition of the release of proapoptotic mitochondrial proteins, sequestration and modulation of the proapoptotic ced-4 protein and its mammalian homologue, and inhibition of a direct cytotoxic effect of the proapoptotic regulators Bax and Bak (Haunstetter and Izumo, 1998). The Bax protein promotes cardiomyocyte apoptosis by directly inducing the release of cytochrome C from mitochondria (Jurgensmeier et al., 1998). Translocation of Bax from the cytosol to the mitochondria increases the permeability of the outer mitochondrial membrane and the release of cytochrome C and other apoptosis-inducing factors, probably inducing pore formation in the mitochondrial membrane (Jurgensmeier et al., 1998).

In the present study, the effects of DADLE on survivin and Bcl-2 expression appeared after 6 hours under DEPV conditions, whereas the effects of DADLE on ERK

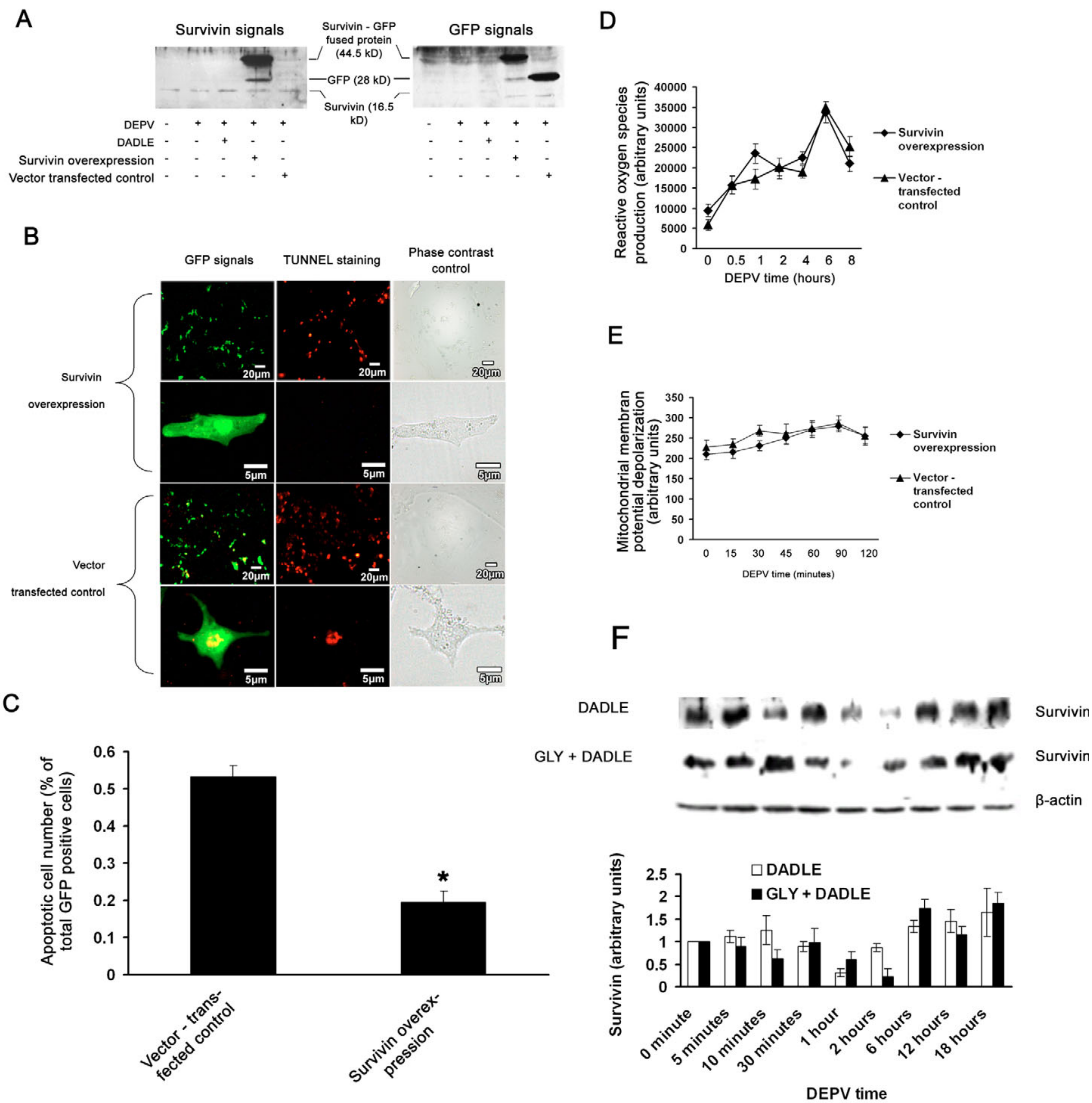

Fig. 9. Survivin overexpression protected DEPV-induced apoptosis but not ROS production and mitochondrial membrane depolarization. (A) Western blots analysis showing high levels of the GFP-survivin fusion protein in the cardiomyocytes transfected with GFP-survivin vectors but not in the cells transfected with GFP control vector. (B,C) Representative micrographs (B) and quantification of apoptotic cardiomyocytes expressed as the ratio of the TUNEL positive cells to GFP positive cells (C). (D,E) Survivin overexpression had no effect on ROS production (D) and mitochondrial membrane potential depolarization (E). (F) Glybenclamide was not effective in preventing DADLE-induced increase in survivin expression. Values are the mean ± s.d. **P*<0.05. *n*=6 in each group. DEPV, serum and glucose deprivation; DADLE, [D-Ala2, D-Leu5]-enkephalin acetate salt; GFP, green fluoresce protein; TUNEL, TdT-mediated dUTP nick end labeling; ROS, reactive oxygen species.

phosphorylation and Bax translocation were apparent after 2 hours. However, the data provided do not yet establish a causal relationship between survivin, Bcl-2 and Bax actions. The Bcl-2- and Bax-dependent pathways were probably involved in addition to the survivin-dependent pathway in DADLE-induced cardiomyocyte protection.

On the other hand, activation of ERK1/2 has been demonstrated to promote myocyte hypertrophy and, to protect the myocyte against ischemic injuries and apoptosis (Bishopric et al., 2001; Bueno and Molkentin, 2002). Pharmacological approaches, such as treatment with opioids have been shown in the rat to induce a persistent activation of ERK1/2 as well as provide protection to the heart against a prolonged ischemic insult (Fryer et al., 2001b). In our present study, DADLE pretreatment induced a late-onset and long-lasting phosphorylation of ERK1/2. We suggest, on the basis of the finding that the ERK1/2 inhibitors abolished DADLE-induced changes in survivin, Bcl-2 and Bax, that ERK1/2 acts as an upstream regulator of survivin, Bcl-2 and Bax.

Moreover, the present study showed that DADLE also prevented the earlier apoptotic event, e.g. mitochondrial membrane damage, after 45 minutes under DEPV conditions. This protective effect of DADLE might be ascribed to an opening of the K_{ATP} channels in view of the observation that this effect was prevented by the K_{ATP} channel inhibitor glybenclamide. However, glybenclamide was not able to block DADLE-induced upregulation of survivin. On the other hand, overexpression of survivin did not prevent the rather early mitochondrial membrane damage, though the late cardiomyocyte apoptosis was prevented by survivin overexpression. Thus, mitochondrial membrane protection and upregulation of survivin belong to two independent pathways which might both mediate the anti-apoptotic effect of DADLE.

In addition, ROS have been shown to promote cardiomyocyte apoptosis (Xia et al., 2006). In the present study, DADLE prevented DEPV-induced ROS production. On the basis of these data, we hypothesize that a ROS pathway may be involved in the anti-apoptotic actions of DADLE. However, this ROS pathway appears to be independent of survivin actions in view of the finding that survivin overexpression had no effect on the DEPV-induced ROS production.

As summarized in Fig. 10, survivin mediates the anti-apoptotic effect of δ-opioid receptor activation in cardiomyocytes. ERK1/2 and PI 3-kinase are upstream regulators of survivin. In addition, the anti-apoptotic effect of δ-opioid receptor activation is also associated with a protection of the mitochondrial membrane, a reduction in ROS production, an increase in Bcl-2 expression and a decrease in Bax translocation.

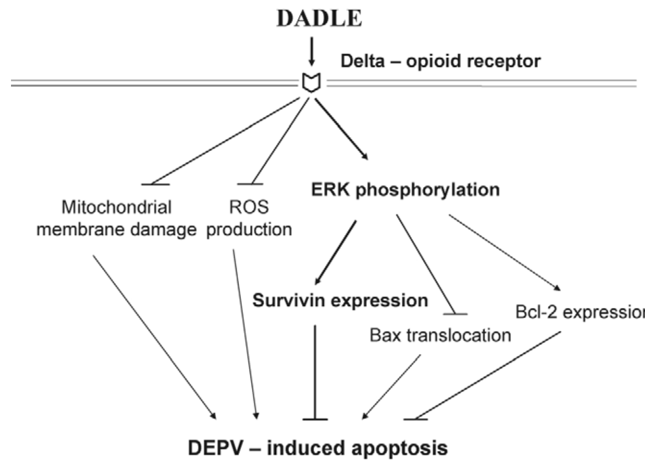

Fig. 10. Schematic illustration of the intracellular signaling mechanisms of the anti-apoptotic effect of DADLE shown in the present study.

Materials and Methods

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

DEPV-induced apoptosis in cultured rat neonatal cardiac myocytes

Primary cultures of neonatal cardiac myocytes were prepared according to the methods described previously (Wang et al., 2002). Briefly, cardiac cells were dissociated from 1-day-old Sprague-Dawley (SD) rat pups with 0.1% trypsin. To selectively enrich for myocytes, dissociated cells were plated and kept at 37°C with 5% CO_2 for 1 hour. The non-myocytes attached readily to the bottom of the culture dish. The resultant suspension of cardiomyocytes were plated into 60 mm^2 culture dishes at a density of 5×10^5 cells/ml and cultured in humidified 5% CO_2, 95% air at 37°C. 5-Bromo-2′-deoxyuridine (100 μM) was added during the first 48 hours to prevent proliferation of non-myocytes. Using this method, we routinely obtained cultures with ~95% myocytes, as assessed by microscopic observation of spontaneous cell beating. All experiments were performed using cells cultured for day 3. To establish the serum and glucose deprivation (DEPV) model, the cells were cultured in a 1:1 mixture of DMEM and Ham F12 without glucose and serum for the indicated time periods.

Experimental protocols

As shown in Fig. 1, cardiomyocytes cultured under DEPV conditions for 5 minutes to 18 hours were pretreated with morphine (1 μmol/l) in the presence or absence of the non-specific opioid receptor antagonist naloxone (NAL; 5 μmol/l), the κ-opioid receptor antagonist guanidinyl-naltrindole (GNTI; 5 μmol/l), or the δ-opioid receptor antagonist natrindole (NAT; 5 μmol/l). The cardiomyocytes were also pretreated with the specific δ-opioid receptor agonist [D-Ala2, D-Leu5]-enkephalin acetate (DADLE; 10 μmol/l) in the presence or absence of natrindole (5 μmol/l), 2′-amino-3′-methoxyflavone (PD98059; 50 μmol/l), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene (U0126; 10 μmol/l), or 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002; 10 μmol/l), or an K_{ATP} antagonist glybenclamide (GLY; 1 μmol/l). Both PD98059 and U0126 were used as inhibitors of ERK1/$_2$. U0126 was used as an inhibitor of both MEK1 and MEK2 whereas PD98059, which is a better inhibitor of MEK1 than MEK2, was used preferentially for inhibition of MEK1. LY294002 was used as an inhibitor of phosphoinositol 3-kinase (PI 3-kinase). The antagonists or inhibitors were given 30 minutes or 5 minutes before the administration of the opioid receptor agonists. The control group was cultured in DMEM-F12 with low glucose (1000 mg/l) and 2% serum and treated with vehicles. The DADLE-treated groups were also studied in the presence or absence of survivin siRNA (see the following section 'transfection of siRNAs'). Some cells were transfected and overexpressed with vectors carrying the survivin gene or with control vectors (see 'survivin overexpression').

Ultramicroscopic observation

Cultured neonatal rat cardiac myocytes were detached from the culture dishes and collected by centrifugation. The cells were fixed, dehydrated, infiltrated and embedded in epoxy resin. Ultra thin sections of 50-60 nm were cut using a LKB-1 ultramicrotome and stained before they were observed and photographed using a JEM-1200 EX transmission electron microscope (Wang et al., 2002).

Measurement of intracellular ROS generation

The oxidation-sensitive probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma, USA) was used for the analysis of the generation of intracellular reactive oxygen species (ROS). The cells were incubated with DCFH-DA (10 μmol/l) at 37°C for 30 minutes. The ROS production was assessed on the basis of the fluorescence intensity at an excitation wavelength at 488 nm and an emission wavelength at 525 nm.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was assessed by using the MitoCapture Mitochondrial Apoptosis Detection kit according to the protocols provided by the manufacturer (BioVision, Tokyo, Japan). MitoCapture is a cationic dye that aggregates in the mitochondria and gives off a bright red fluorescence in healthy cells. In apoptotic cells, MitoCapture fails to aggregate in the mitochondria as a result of altered mitochondrial transmembrane potential and remains in the cytoplasm in its monomer form, which may exhibit green fluorescence. Briefly, the cells were incubated in 1 ml incubation buffer containing 1 μl MitoCapture for 15 minutes at 37°C and 5% CO_2. After staining, the fluorescent signals were measured using a TECAN multifunction microplate reader. The red fluorescence were excited at 530 nm and detected at 630 nm, and the green fluorescence was excited at 488 nm and detected at 530 nm. Cardiomyocyte mitochondrial damage was assessed by examining mitochondrial membrane depolarization, which was indicated by the ratio of red and green signals in the micrographs.

Hoechst 33258 staining
The cultured neonatal rat cardiomyocytes were fixed and stained with Hoechst 33258. The apoptotic cells were identified according to morphological features such as cell shrinkage, membrane blebbing and chromatin condensation, which were observed with a fluorescence microscope (Leica TCS SP2, Wetzlar, Germany).

DNA ladder formation
DNA ladder formation was observed by agarose gel electrophoresis. The cardiomyocytes were lysed in lysis buffer and electrophoresed on 1.5% agarose gel. After staining with ethidium bromide, DNA ladder formation was visualized and photographed under ultraviolet light.

TdT-mediated dUTP nick end labeling
Detection of apoptotic myocytes was also performed by using TdT-mediated dUTP nick end labeling (TUNEL) with a cell apoptosis detection kit (Roche, Applied science, USA). Briefly, after the cells were fixed and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, 50 μl TUNEL reaction mixture was added to samples and the cells, on slides, were incubated in a humidified atmosphere for 60 minutes at 37°C, in the dark. The slides were rinsed three times with PBS and observed using fluorescence microscopy. The percentage of apoptotic cells in each culture dish was calculated by counting condensed or disintegrated nuclei from six randomly selected fields (magnification ×200).

RNA extraction and real-time PCR analysis
Total RNA was extracted from the cultured cells, quantified by absorbance at 260 nm, normalized, and reverse-transcribed into first-strand complementary DNA (cDNA). cDNA mixture (0.4 μg) obtained from the reverse transcription reaction was used in the PCR reaction with a total volume of 50 μl containing 5 μl 10× PCR buffer, 3.5 mmol/l $MgCl_2$, 0.32 mmol/l of each primer, 1 μl 10 mmol/l dNTP and 0.16 mmol/l probe. Bax and Bcl-2 mRNA expression was determined with quantitative real-time PCR using iCycler iQ™ real-time PCR Detection System (Bio-Rad, Hercules, CA USA). To minimize and control the sample variations, mRNA expression of the target gene were normalized relative to the expression of the housekeeping gene GAPDH. The probes for the target genes were labeled with 6-carboxy-fluorescein (FAM) reporter dye at the 5′ end and 6-carboxy-tetramethyl-rhodamine (TAMRA) dye as the quencher at the 3′ end. For the Bax gene, the forward primer was 5′-CCCACCAGCTCTGAACAGTT-3′, the reverse primer was 5′-TTGTCCAGTTCATCTCCAATTCG-3′ and the probe was FAM-5′-CGG-AGACACTCGCTCAGCTTCTTGGT-3′-TAMRA, corresponding to positions 37, 179 and 191, respectively. For the Bcl-2 gene, the forward primer was 5′-CACCCCTGGCATCTTCTCCT-3′, the reverse primer was 5′-CGACGGTAG-CGACGAGAGAA-3′ and the probe was FAM-5′-CCAGCCTGAGAGCAACCG-AACGCC-3′-TAMRA, corresponding to positions 366, 189 and 387, respectively. For the GAPDH gene, the forward primer was 5′-GGACCTGACCTGCCGTCTAG-3′, the reverse primer was 5′-TAGCCCAGGATGCCCTTGAG-3′ and the probe was FAM-5′-TCCGACGCCTGCTTCACCACCTTCT-3′-TAMRA, corresponding to positions 753, 851 and 824, respectively, of the published sequence (Berra et al., 2000). For the survivin gene, the forward primer was 5′-GGAGGCTGG-CTTCATCCCAC-3′, the reverse primer was 5′-GGAACCGGATGACAACCC-3′ and the probe was FAM-5′-CATCCACTGCCCTACCGAGAATGAGC-3′-TAMRA, corresponding to positions 121, 208 and 133, respectively.

Two-step real-time PCR denaturing, annealing and extension reactions was performed for 45 cycles of 30 seconds at 95°C, 1 minute at 60°C (for Bax, Bcl-2, survivin and GAPDH). Increasing curves of reporter dye fluorescence emission were recorded and analyzed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) to determine the threshold cycle (Ct) value. Each sample was run and analyzed in triplicate and the Ct values for survivin, Bax and Bcl-2 were subtracted from the Ct value of GAPDH to yield ΔCt values. The average ΔCt was calculated for the control group and this value was subtracted from the ΔCt of all other samples (including the control group). This resulted in a ΔΔCt value for all samples, which was then used to calculate the fold induction of the mRNA levels of survivin, Bax and Bcl-2 using the formula $2^{-\Delta\Delta Ct}$, as recommended by the manufacture (Bio-Rad Hercules, CA, USA).

Western blot analysis
Protein was extracted from the cultured neonatal rat cardiomyocytes. Protein samples (50 μg) were separated by 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Gelman-Pall, Ann Arbor, MI, USA), blocked with 5% non-fat milk, and incubated with either rabbit polyclonal antibodies against phosphorylated ERK1/2 (1:1000), or rabbit polyclonal antibodies against ERK1/2 (1:1000; Cell Signaling Technology, MA) or mouse monoclonal antibodies against Bcl-2 (1:1000; Sigma, St Louis, MO) or mouse monoclonal antibodies against Bax (1:1000; Sigma), or rabbit polyclonal antibodies against survivin (1:500; Santa Cruz, CA) respectively, at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:1000) or anti-rabbit IgG (1:1000) (all from Calbiochem, CA) for 1 hour at room temperature. Chemiluminescent signals were generated by the addition of the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and detected on a radiographic film.

Translocation of Bax from cytosol to mitochondria was determined by detection of the differential distribution of the protein in cytosol and in mitochondria. To measure the Bax protein, the cultured cardiomyocytes were scraped from the culture dishes and centrifuged at 800 ***g*** for 5 minutes. The cells were resuspended in 150 μl of cold lysis buffer containing (in mmol/l, at pH 7.5) 250 sucrose, 20 HEPES, 10 KCl, 1 $MgCl_2$, 1 EDTA, 1 EGTA, 1 dithiothreitol, 1 phenylmethanesulfonyl fluoride, and were incubated for 5 minutes on ice. The cells were homogenized with several strokes with a pestle and then the suspension was centrifuged at 750 ***g*** for 10 minutes at 4°C to precipitate the nuclei. The supernatant was centrifuged at 12,000 ***g*** for 10 minutes at 4°C to collect the mitochondrial fraction. The supernatant was further centrifuged at 100,000 ***g*** for 60 minutes at 4°C, and the resultant supernatant was collected as the cytosolic fraction (Tatsumi et al., 2003).

Transfection of siRNAs
All siRNAs and control nucleic acids were designed and provided by Shanghai Genechem Co., Ltd. The siRNAs were designed according to the sequences provided by GenBank. For targeting the survivin gene, three rBIRC5-specific double-stranded siRNAs (sense, 5′-CCGAGAAUGAGCCUGAUUU-3′; antisense, 5′-AAAUCAGGCUCAUUCUCGG-3′) were synthesized. siRNA for GAPDH (sense, 5′-GUGGACAUUGUUGCCAUCA-3; antisense, 5′-UGAUGGCAACA-AUGUCCAC-3′) and one irrelevant nucleic acid (sense, 5′-UUCUCCGA-ACGUGUCACGU-3′; antisense, 5′-ACGUGACACGUUCGGAGAA-3′) were used to exclude any non-specific effect of nucleic acid transfection on the cells.

Neonatal cardiomyocytes were maintained in DMEM-F12 medium containing 10% fetal bovine serum and antibiotics and transfected using TransMessenger™ Transfection Reagent following the instructions of the manufacturer (Qiagen, Hilden, Germany). Briefly, Enhancer R was first diluted in buffer EC-R and then mixed with siRNA in the indicated amounts. The ratio (w/w) between TransMessenger™ Transfection Reagent and RNA was 4:1. Antibiotic-free transfection medium (OPTI-medium, Gibco, Los Angeles, USA), at 1 ml per well, was added to 6-well plates. Following 20 minutes incubation at 37°C, the RNA-TransMessenger™ Transfection Reagent mixture was added to OPTI-medium to replace the cell culture medium. The cells were maintained in a 37°C incubator with 95% O_2 and 5% CO_2. The medium with siRNAs was replaced by DMEM-F12 medium 24 hours after transfection. Knockdown of gene expression was identified by real-time PCR or western blot analysis 48 hours after transfection of the siRNAs.

Recombination of survivin-expressing vectors and transfection
Full length rat survivin cDNA (Wang et al., 2004) was subcloned into pEGFP-N3 (Clontech Laboratories, Inc.) with *Xho*I and *Eco*RV restricted sites. Cardiomyocytes were cultured in DMEM medium containing 10% fetal bovine serum and antibiotics, and transfected using Lipofectamine 2000 following the instruction of the manufacturer (Invitrogen, California, USA). Lipofectamine 2000 was first diluted in OPTI-MEM I (Gibco-BRL, USA) and then mixed with plasmid DNA. The ratio (w/w) between Lipofectamine 2000 and DNA was 4:1. One and 2 ml medium per well were used for 12- and 6-well plates, respectively. Following a 20-minute incubation period at room temperature, the DNA-Lipofectamine 2000 mixture was added to the antibiotic-free transfection medium (DMEM containing glutamine + 10% FBS) and the culture medium was replaced with this transfection medium. The cells were maintained in a 37°C incubator with 95% O_2, 5% CO_2 until analysis. The medium was replaced with vector free medium 24 hours after transfection.

Statistical analysis
Quantitative data are presented as the mean ± s.d. The mRNA and protein levels were normalized to the mean of control data in each set of experiments. Comparison between the groups was performed by one-way analysis of variance (ANOVA), followed by Tukey's test. In all cases, a P value of <0.05 was taken to indicate statistical significance.

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